Melatonin synthesis: arylalkylamine *N*-acetyltransferases in trout retina and pineal organ are different

Ahmed Benyassi,[1,2] Christian Schwartz,[1,3] Steven L. Coon,[3] David C. Klein[3] and Jack Falcón[1,3,CA]

[1]Laboratoire de Neurobiologie Cellulaire et Neuroendocrinologie, CNRS UMR 6558, Université de Poitiers, 86022 Poitiers-Cedex, France; [2]Faculté des Sciences de Fés, Laboratoire de Physiologie Animale, Fés, Morocco; [3]Section on Neuroendocrinology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-4480, USA

[CA,3]Corresponding Author and address

Received 13 October 1999; accepted 7 november 1999

Acknowledgements: The study was supported by the CNRS, University of Poitiers, and Fondation Langlois (Rennes). We thank Françoise Chevalier, Gaël Epistolin and Nathalie Girard for their technical assistance.

Serotonin *N*-acetyltransferase (AANAT) is the first enzyme in the conversion of serotonin to melatonin. Changes in AANAT activity determine the daily rhythm in melatonin secretion. Two AANAT genes have been identified in the pike, pAANAT-1 and pAANAT-2, expressed in the retina and in the pineal, respectively. The genes preferentially expressed in these tissues encode proteins with distinctly different kinetic characteristics. Like the pike, trout retina primarily expresses the AANAT-1 gene and trout pineal primarily expresses the AANAT-2 gene. Here we show that the kinetic characteristics of AANAT in these tissues differ as in pike. These differences include optimal temperature for activity (pineal: 12°C; retina: 25°C) and relative affinity for indoleethylamines compared to phenylethylamines. In addition, retinal AANAT exhibited substrate inhibition, which was not seen with pineal AANAT. The kinetic differences between AANAT-1 and AANAT-2 appear to be defining characteristics of these gene subfamilies, and are not species specific. *NeuroReport* 11:255–258 © 2000 Lippincott Williams & Wilkins.

Key words: Arylalkylamine *N*-acetyltransferase; Fish; Pineal organ; Retina

INTRODUCTION

In fish, the photoreceptor cells of the retina and of the pineal organ produce melatonin on a rhythmic 24 h schedule [1]: biosynthesis and release are usually low during the day and high at night. These rhythms result from changes in the activity of acetyl CoA:arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87), which converts serotonin to *N*-acetylserotonin [2,3]. There are two AANAT genes in fish, including pike [4] and trout [5,6], termed AANAT-1 and AANAT-2. Analysis of pike tissues and of recombinant pike AANAT-1 and AANAT-2 indicates that AANAT-1 is preferentially expressed in the retina and AANAT-2 is preferentially expressed in the pineal organ; in addition, the enzymes encoded by these genes have distinctly different kinetic characteristics, including different temperature optima and different relative affinities for indoleethylamines and phenylethylamines [4,7]. AANAT-1 exhibits substrate inhibition, whereas AANAT-2 does not [4,7].

We are generally interested in the mechanisms which regulate AANAT activity and have a special interest in analysis of trout AANAT because it differs from that of pike and most other species. In most vertebrates, an internal clock is involved in the regulation of AANAT activity [1]. However, the trout represents a relatively uncommon regulatory system because no clock is involved [1]: AANAT activity appears to be regulated entirely by environmental lighting and does not exhibit a circadian rhythm in constant darkness. These and other differences in regulation have stimulated us to initiate an analysis of the regulation of trout AANAT activity in the pineal organ and retina. As indicated above, AANAT activity in the pike pineal organ, encoded by AANAT-2, differs from that in the retina encoded by AANAT-1 [5]. Accordingly, to study AANAT activity in the trout pineal organ and retina it is necessary to first characterize the enzymes expressed in these tissues. The results of this effort are presented here.

MATERIALS AND METHODS

Animals: Female rainbow trout (*Oncorhynchus mykiss*, Teleost) with body weights ranging from 250 to 300 g, originated from a commercial hatchery. They were main-

tained in oxygenated recycled filtered pond-water under conditions resembling their natural habitat with respect to temperature and illumination (1000 lux intensity at the water surface). The animals were placed in the dark at midday and killed 6 h later under dim red light. The pineal organs and retinas were removed, frozen in liquid nitrogen and stored at −78°C.

AANAT activity assay: Typically, each experiment used 20 pineal organs or five retinas. Pooled organs were sonicated in 0.2 M phosphate buffer (pH 6). Proteins were quantified in an aliquot of the homogenate [8]. Homogenates were then centrifuged for 30 s at 5000 × ***g*** and 4°C. Supernatants were kept on ice (no more than 5 min) until assayed for AANAT. The assay is a modification of a published method [7]. Typically a 50 µl sample of the homogenate (i.e. 100–150 µg pineal proteins or 400-500 µg retinal proteins) was added to 50 µl of a solution containing tryptamine (Sigma, France), acetyl-CoA (Boehringer Mannheim, France) and [^{3}H]acetyl-CoA (Dupont NEN, France; final sp. act. 4 mCi/mmol). The incubation time was 30 or 40 min. Other conditions are described in Results and in figure legends. Preliminary studies indicated that the optimal temperature for pineal AANAT activity was 12°C, and that for retinal AANAT activity was 25°C. Accordingly, the corresponding temperatures were used to assay the enzymes from these tissues. The reaction was stopped by the addition of 1 ml chloroform (4°C). Acetylated products were extracted, and radioactivity was counted, as described previously [7]. All data are presented as the mean of duplicate determinations from one representative experiment (out of 2 or 3); statistical analysis and curve fitting has been detailed elsewhere [7].

RESULTS

Linearity of AANAT activity as a function of protein concentration (not shown): Pineal AANAT activity (pmol/h of [^{3}H]acetyl-tryptamine formed) was linear with homogenate protein amounts ranging from 20 to 160 µg; retinal AANAT activity increased linearly with protein concentrations ranging from 150 to 500 µg.

Linearity of AANAT activity with time (Fig. 1): After an initial 10–15 min lag, pineal AANAT activity and retinal AANAT activity increased linearly with increasing incubation times up to 60 min (pineal, $r^2 = 0.96$, slope = 0.18 ± 0.01) or to 120 min (retina, $r^2 = 0.99$, slope = 0.015 ± 0.0003). The reasons for the presence of a lag phase before the steady-state rate was obtained have been extensively discussed elsewhere [9].

Temperature of incubation (Fig. 2): AANAT activity was assayed at temperatures ranging from 4°C to 50°C. Pineal homogenates showed a relatively sharp maximum at about 10–12°C. Retinal homogenates displayed a broader optimum temperature of around 25°C.

Influence of buffer molarity and pH (not shown): AANAT activity was 15-fold (retina) and 2-fold (pineal) higher in 0.2 M phosphate buffer than in 0.1 M buffer. A further increase in molarity resulted in a 2-fold (0.3 M) to a 4-fold (0.4 M) decrease in enzyme activity. The effects of pH were measured between pH 5.5 and 8, at a fixed molarity of 0.2 M. Activity was the highest at a pH of 6 in both tissues and was nearly undetectable at pH 7 and above.

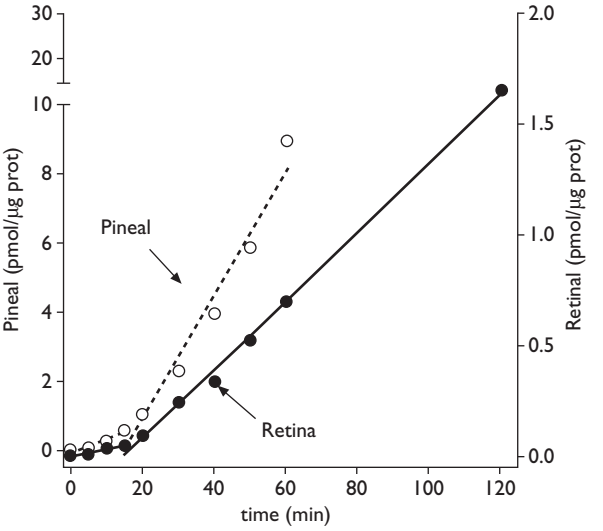

Fig. 1. AANAT activity as a function of time. Homogenates were incubated in the presence of 0.5 mM acetyl-CoA and 4 mCi/mmol [^{3}H]acetyl-CoA, at 25°C and 0.3 mM tryptamine (retina) or 12°C and 10 mM tryptamine (pineal).

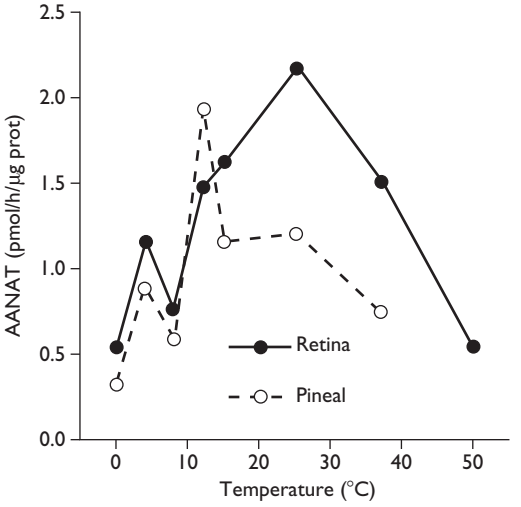

Fig. 2. AANAT activity as a function of temperature. Pineal and retinal homogenates were incubated for 40 min in the presence of 0.5 mM acetyl-CoA and 4 mCi/mmol [^{3}H]acetyl-CoA. Tryptamine concentration was 0.3 mM (retina) or 10 mM (pineal).

AANAT activity as a function of acetyl-CoA concentration (Fig. 3): Acetyl-CoA concentration was varied in the presence of 0.3 mM (retina) or 10 mM (pineal organ) tryptamine. The resulting experimental data fit the Michaelis–Menten equation both in the retina and in the pineal. These values were in good agreement with those obtained after transformation of the data by plotting S/V = f(S), both in the retina (Vmax = 1.79 ± 0.14 pmol/h/µg protein, Km = 0.09 ± 0.03 mM) and in the pineal (Vmax = 1.19 ± 0.07

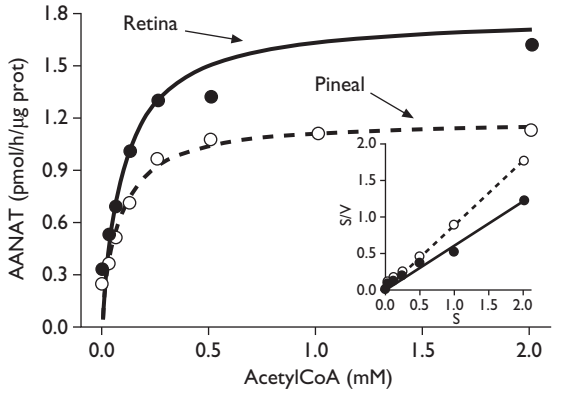

Fig. 3. AANAT activity as a function of acetyl-CoA concentration. Homogenates were incubated for 40 min at 25°C and 0.3 mM tryptamine (retina) or 12°C and 10 mM tryptamine (pineal). Inset: S/V against S plot.

pmol/ h/µg protein, Km = 0.07 ± 0.017 mM); after transformation a single straight line was obtained ($r^2 = 0.99$ for both the retina and the pineal).

AANAT activity as a function of tryptamine concentration (Fig. 4): Data for the pineal organ fitted poorly to the Michaelis–Menten equation ($r^2 = 0.86$). Transformation according to the S/V = f(S) function suggested the presence of two components. The first had an apparent Vmax and Km of 0.83 ± 0.05 pmol/h/µg protein and 0.08 ± 0.05 mM, respectively; the second had an apparent Vmax of 4.08 ± 0.05 pmol/h/µg protein and an apparent Km of 7.8 ± 0.16 mM.

In the retina, AANAT activity as a function of tryptamine concentration did not follow the Michaelis–Menten equation. After an initial increase in activity up to 0.3 mM tryptamine, further increase in tryptamine concentration resulted in a decreased activity up to 2.5 mM; activity did not change significantly at higher tryptamine concentrations. The apparent Vmax and Km, deduced from the S/V = f(S) transformation of the data were 4.86 ± 0.108 pmol/h/µg protein and 0.19 ± 0.42 mM, respectively. The result of the transformation of the data according to the 1/V = f(1/S) function suggested competitive inhibition at high substrate concentration (not shown). The inhibition constant was estimated from the exponential decay part of the curve in Fig. 4; the apparent Ki was 1.16 ± 0.34 mM. When the apparent Km and Ki were fixed at the above values, the equation of Haldane fit the experimental data before the plateau was reached but not after, suggesting that inhibition was never total. The equation of Haldane accounts for an inhibition by high substrate concentration [9].

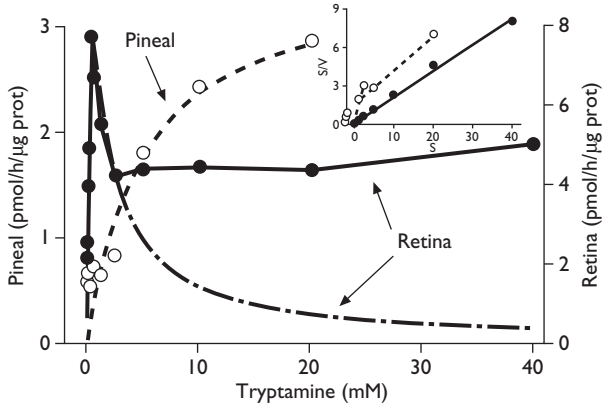

Fig. 4. AANAT activity as a function of tryptamine concentration. Homogenates were incubated for 40 min at 25°C (retina) or 12°C (pineal), in the presence of 0.5 mM acetyl-CoA and 4 mCi/mmol [^{3}H]acetyl-CoA. The dash-dot-dash line, associated with the retinal data, corresponds to the hypothetical curve obtained when the data were fitted to the Haldane equation (V = Vmax/(1 + Km/S+S/Ki)) using the Km and Ki estimates as indicated in the text.

AANAT activity as a function of phenylethylamine concentration (Fig. 5): Acetylation of phenylethylamine (PEA) by pineal homogenates was not detected. Conversely, acetylation of PEA in the presence of retinal extracts followed the Michaelis–Menten equation (Vmax = 6.73 ± 0.22 pmol/h/µg protein, Km = 5.24 ± 0.53 mM).

DISCUSSION

The studies reported here are important for two reasons. First, they indicate that the characteristics of trout and pike AANAT-1, the enzyme expressed in the retina of these species, are generally similar; and that those of trout and pike AANAT-2, the enzyme expressed in the pineal organs of these species are similar (Table 1). This indicates that the reported differences between pike AANAT-1 and AANAT-2 [4,5] do not reflect unique species differences. Rather, these differences are also seen in the trout, and appear to represent reliable identifying characteristics of the proteins encoded by the different genes.

The second reason these studies are important are that they establish a distinctly different set of optimal assay conditions for the detection of trout AANAT-1 and for detection of trout AANAT-2 activity (Table 1). The most notable differences are seen in the following characteristics: relative ability to acetylate phenylethylamine, optimal pH, optimal temperature, optimal buffer concentration, and

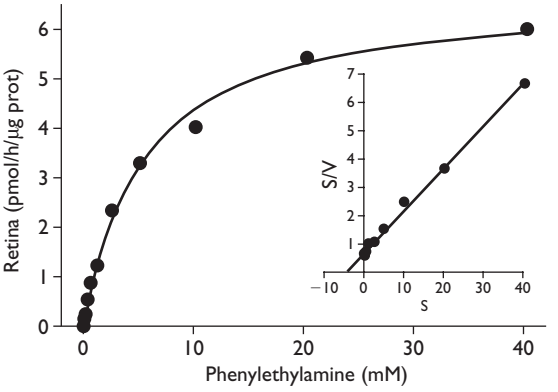

Fig. 5. AANAT activity as a function of phenylethylamine concentration. Retinal homogenates were incubated (40 min) at 25°C in the presence of 0.5 mM acetyl-CoA and 4 mCi/mmol [^{3}H]acetyl-CoA. Inset: S/V against S plot.

Table 1. Comparison of the kinetic characteristics of arylalkylamine *N*-acetyltransferases (AANAT-1 and AANAT-2) in the trout and pike.

	AANAT-1			AANAT-2		
	Trout retina	Pike retina [4]	Pike recombinant [7]	Trout pineal	Pike pineal [4]	Pike recombinant [7]
Optimum pH	6	6	nd	6	6	nd
Optimum molarity (M)	0.2	0.2	nd	0.2	0.2	nd
Optimum temperature (°C)	25	37	37	12	18	18
Km Acetyl-CoA (mM)	0.09	0.1	nd	0.07	0.3	nd
Acetylation						
Tryptamine	+	+	+	+	+	+
PEA	+	+	+	−	−	−
Km (mM)						
Tryptamine	0.19	0.12	0.02	7.8	7.5	7
PEA	5.24	nd	0.9	na	na	na
Substrate inhibition						
Tryptamine	+	+	+	−	−	−
PEA	−	−	−	na	na	na

PEA: phenylethylamine; nd: not determined; na: not applicable.

substrate inhibition with tryptamine. With these established, it will be possible to initiate analysis of the physiological regulation of trout pineal and retinal AANAT activity in a meaningful manner.

CONCLUSION

The first indication that two AANAT enzymes are present in vertebrates was obtained in the pike [4,7]. This study adds to previous information suggesting that a similar situation occurs in the trout [5,6], making it clear that the dominant form of AANAT expressed in the trout retina belongs to the AANAT-1 gene subfamily and that expressed in the trout pineal organ belongs to the AANAT-2 subfamily (Table 1). This study has identified ideal conditions to assay AANAT activity in the trout pineal organ and retina, which differ significantly in temperature and tryptamine concentration. This information will be useful in future studies of the regulation of AANAT activity in the trout. The differences in characteristics of the forms of enzymes expressed in the pineal organ and retina might be related to the fact that ocular melatonin serves local functions, whereas pineal melatonin has neurohormonal properties.

REFERENCES

1. Falcon J. *Prog Neurobiol* **58**, 121–162 (1999).
2. Klein DC, Auerbach DA, Namboodiri MA *et al*. Indole metabolism in the mammalian pineal gland. In: Reiter RJ, ed. *The Pineal Gland*. Boca Raton: CRC Press, 1981: 199–227.
3. Klein DC, Coon SL, Roseboom PH *et al*. *Recent Prog Horm Res* **52**, 307–357 (1997).
4. Coon SL, Bégay V, Deurloo D *et al*. *J Biol Chem* **274**, 9076–9082 (1999).
5. Bégay V, Falcón J, Cahill G *et al*. *Endocrinology* **139**, 905–912 (1998).
6. Mizusawa K, Iigo M, Suetake H *et al*. *Zool Sci* **13**, 345–351 (1998).
7. Falcón J, Bolliet V and Collin JP. *Pflügers Arch* **342**, 386–396 (1996).
8. Bradford MM. *Anal Biochem* **72**, 248–254 (1976).
9. Tipton KF. Principles of enzyme assay and kinetic studies. In: Eisenthal R and Danson MJ, eds. *Enzyme Assays, a Practical Approach*. Oxford, IRL press, 1992: 1–58.